Review

The good, the bad and the ugly in oxygen-sensing: ROS, cytochromes and prolyl-hydroxylases

Till Acker a, Joachim Fandrey b, Helmut Acker b,*

a Neurologisches Institut (Edinger Institut), Klinikum der J.W. Goethe Universität Frankfurt am Main, Deutschordenstr. 46, D-60528 Frankfurt, Germany
b Institut für Physiologie, Universität Duisburg-Essen, Hufelandstr. 55 IG1, D-45147 Essen, Germany

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Abstract

Current concepts of cellular oxygen-sensing include an isoform of the neutrophil NADPH oxidase, different electron carrier units of the mitochondrial electron transport chain (ETC), heme oxygenase-2 (HO-2), and a subfamily of 2-oxoglutarate dependent dioxygenases termed HIF (hypoxia inducible factor) prolyl hydroxylases (PHDs) and HIF asparagyl hydroxylase FIH-1 (factor-inhibiting HIF). Different oxygen sensitivities, cell-specific distribution and subcellular localization of specific oxygen-sensing cascades involving reactive oxygen species (ROS) as second messengers may help to tailor various adaptive responses according to differences in tissue oxygen availability. Herein, we propose an integrated model for these various oxygen-sensing mechanisms that very efficiently regulate HIF-α activity and plasma membrane potassium-channel (PMPC) conductivity.

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1. Physiology of oxygen-sensing

The respiratory and cardiovascular systems ensure the uptake and appropriate distribution of oxygen to serve as the terminal electron acceptor in mitochondria to generate ATP. Extraction of oxygen from the environment and its distribution not only for oxidative phosphorylation but likewise as a substrate for other biochemical reactions has been conserved through evolution by the development of advanced multi-level systems. These systems tightly maintain O2 homeostasis allowing the cell to survive, function and thrive in regions with heterogeneous tissue oxygen tension (PO2) distribution as shown in the lower part of Fig. 1 [11]. Impairment in oxygen supply elicits a left shift of the tissue PO2 distribution (see lower part of Fig. 1) inducing different cellular responses of which the “mirror image” PO2 sensitivity of the phosphoenol pyruvate carboxykinase and glucokinase gene in primary rat liver cells is a good example [59]. To achieve such responses cells need an oxygen-sensor system which has to fulfill several crucial requirements. Sensor responses should inherently depend on the ability to sense oxygen concentrations and, whenever the PO2 deviates from a given preset value, should initiate distinct signaling cascades. The wide range of the tissue PO2 distribution suggests that the threshold of activation may vary from organ to organ and cell to cell. Thus, the oxygen sensor(s) should be flexible and highly adaptive allowing for graded cellular responses. Oxygen-sensing heme proteins like complex III or IV of the respiratory chain or isoforms of the NADPH oxidase, and non-heme oxygen-sensing proteins like PHDs or HO-2 have been described as candidate sensor systems for the...
regulation of hypoxia inducible responses (see upper part of Fig. 1) [1].

2. Hypoxia inducible factors (HIFs)

The identification of the HIF transcription factor [104] was a milestone in our understanding of oxygen physiology. The HIF complex is a heterodimer composed of constitutively expressed HIF-β and O₂ regulated HIF-α subunits all being members of the bHLH (basic helix loop helix)-PAS (PER-ARNT-SIM) family of transcription factors. Both HIF-α and HIF-β proteins exist as isoforms (HIF-1α, HIF-2α, HIF-3α and HIF-1β=ARNT (aryl hydrocarbon receptor nuclear translocator), ARNT2 and ARTN3, respectively) [2,34,108]. HIF activity is tightly regulated throughout the range of physiological and pathological oxygen concentrations. Predominantly hydroxylation and ubiquitination but also acetylation, S-nitrosylation and phosphorylation have been shown to determine half-life and/or transcriptional activity of HIF [14]. Oxygen dependent enzymatic hydroxylation of proline residues within HIF-α subunits constitutes

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Fig. 1. Integrated oxygen-sensor model: during hypoxia the oxygen distribution curve is more and more shifted leftward to lower PO₂ values resulting in the subsequent activation of low, intermediate and high affinity oxygen sensors. As sensors heme proteins, like complex III or IV of the respiratory chain or isoforms of the NADPH oxidase, and non-heme proteins like PHDs or HO-2 have been described. O₂ consumption by mitochondria, NADPH oxidase and HO-2 influences the PO₂ as well ROS directly produced or generated via a Fenton reaction. This will in the tissue additionally affect PHD activity or HIF stability. We propose that the flexibility and wide operating field of oxygen-sensing is maintained by several oxygen-sensor systems interfacing with and changing PHD and FIH activity via shaping the intracellular PO₂ gradients, ROS levels and co-factor concentrations such as the redox state of iron, vitamin C or 2-oxoglutarate levels. PMPC conductivity is under the control of ROS and CO produced in dependence on PO₂. The diverse interactions as indicated by arrows are described in Synopsis with three different scenarios (adapted from [2]).
Fig. 2. Possible involvement of hydroxyl radicals (OH\(^\cdot\)) – generated in a perinuclear iron-dependent Fenton reaction – in O\(_2\)-dependent gene expression. The putative Fenton reaction was imaged in HepG2 cells using the non-fluorescent DHR 123 together with fusion proteins that allow fluorescent labeling of the perinuclear endoplasmic reticulum (ER). DHR 123 is irreversibly oxidized to fluorescent rhodamine 123 by scavenging OH\(^\cdot\). 3D 2PCLSM demonstrated OH\(^\cdot\) generation (white) in distinct hot spots of perinuclear ER pockets (red). The ER-based Fenton reaction was strictly O\(_2\)-dependent. The left panels show from top to bottom an increasing OH\(^\cdot\) generation starting from the experiment due to the irreversible oxidation of DHR123 from anoxia (0% O\(_2\)), followed by hypoxia (3% O\(_2\)) and normoxia (20% O\(_2\)). The OH\(^\cdot\) generation was drastically enhanced at each PO\(_2\) step by phototoxic blue light irradiation of the cells for 5 s as shown in the right panels and quantified in the inset.
the critical modification that determines protein stability. Oxygen dependent prolyl hydroxylation allows capture by the protein product of the von Hippel Lindau tumor suppressor gene (pVHL), which acts as the recognition component of an E3-ubiquitin ligase enzyme. Subsequent ubiquitination targets the complex for proteosomal degradation. As a consequence, only low-level HIF-α protein if any can be detected in the presence of oxygen. With decreasing oxygen concentrations, however, cellular HIF-α rapidly and exponentially rises. A second oxygen dependent switch involving hydroxylation of an asparagine residue within the C-terminal transactivation domain regulates transcriptional activity most likely by interfering with recruitment of the coactivator p300 under well oxygenated conditions. This results in reduced transcriptional activity of HIF at high $P_O_2$.

The HIF transcriptional system acts as a master regulator of oxygen-regulated gene expression inducing adaptive responses towards hypoxia. So far, more than 70 genes have been identified as potential HIF targets but the list of HIF regulated genes is most likely much longer. Adenoviral mediated HIF-1α induction in endothelial cells upregulated 245 genes and downregulated 325 genes by more than 1.5-fold [70]. The largest category of genes downregulated by hypoxia encoded proteins involved in cell growth/proliferation. Many genes that were upregulated by hypoxia encoded for cytokines/growth factors, receptors, and other signaling proteins. In addition, there is increasing evidence implicating HIF in biological functions that require its activation under normoxic conditions. Among others, mediators of inflammation including nitric oxide, growth factors and vascular hormones regulate the normoxic activation of HIF.

3. Reactive oxygen species (ROS)

In contrast to the well known harmful action of ROS under oxidative stress, kinetics of lower levels of ROS is involved in oxygen-sensing that influences HIF stability [34], PHD activity [18,38,66] and PMPC activity [29]. To address the function of ROS in oxygen-sensing it is crucial to accurately record intracellular ROS kinetics in response to $P_O_2$ changes which is technically demanding. ROS sensitive fluorescence dyes like 2',7'-dihydorhodamine (DHR) or dihydroethidium (DHE) are fluorescent when oxidized by ROS serve as probes for ROS in the cell. However, ROS quantification by ROS sensitive fluorescence dyes is clearly hampered by the following four major drawbacks limiting their applicability:

1. Both dyes DCFH and DHR 123 are oxidized by ROS in an irreversible manner to fluorescent DCF or Rhodamine 123, respectively. A return of the intracellular DCF fluorescence signal to control values after reoxygenation as shown in several publications [20,48,71] can therefore not be related to a decrease in ROS production.

2. Intracellular ROS sensitive fluorescence dyes are only recorded at the emission peak wavelength due to a missing isosbestic point. ROS measurements can therefore not be corrected for changes of intracellular dye concentration. Indeed, it has been shown that DCFH and DCF are both substrates for the multidrug-resistance family (MDR)/transporter associated with antigen processing (TAP) transporter subfamily. In hepatocytes, hypoxia has been shown to downregulate MDR-1B expression. This would result in a reduced outward transport and lead to an intracellular accumulation of DCF under prolonged hypoxia exceeding the normoxic control value severalfold. Upon return to normoxia DCF fluorescence would decrease due to reactivation of the transport [51,58], a phenomenon which could account for the observed DCF normalization after reoxygenation reported by some groups [20,48,71]. An improvement seems to be the use of the dye hydroethidine that reacts with superoxide resulting in the formation of an oxidized product, which binds to DNA and leads to the enhancement of fluorescence [18,26]. Furthermore intracellular ROS-sensitive FRET probes could be a promising new tool [43]. However all these techniques are blurred by the following pitfall.

3. Violet-blue light as used for DCF and rhodamine 123 fluorescence excitation is phototoxic to mammalian cells, and this toxicity has been linked with cellular production of $H_2O_2$ [53]. Two-photon (2P) fluorescence excitation with wavelengths above 800 nm prevents light-induced $H_2O_2$ production in cells, possibly by minimizing photoreduction of flavin-containing oxidases. The use of 2P confocal laser scanning microscopy (2PCLSM) is therefore mandatory to avoid phototoxic ROS production during intracellular ROS measurements [66].

4. Fig. 2 gives a state of the art example on how to image the potential involvement of $OH$- generated from $H_2O_2$ in the presence of Fe(II) in an intracellular Fenton reaction [101]—in $O_2$-dependent gene expression. The putative Fenton reaction was imaged and localized in HepG2 cells using non-fluorescent DHR 123 together with fusion proteins that allow fluorescent labeling of the endoplasmic reticulum (ER). DHR 123 is irreversibly oxidized to fluorescent rhodamine 123 by scavenging $OH$ [31]. 3D 2PCLSM as shown in Fig. 2 demonstrated $OH$ generation (white) in distinct hot spots of perinuclear ER pockets (red). This ER-based Fenton reaction was strictly $PO_2$-dependent [66]. The left panels show from top to bottom an increasing $OH$ generation when starting the experiment from anoxia (0% $O_2$), followed by hypoxia (3% $O_2$) and normoxia (20% $O_2$). The $OH$ generation was further enhanced at each $PO_2$ step by phototoxic blue light irradiation of the cells for 5 s as shown in the right panels. At such short wavelength illumination caused rhodamine 123 fluorescence irrespective of unchanged $PO_2$ values.
4. Oxygen sensitive PMCP

Physiological adaptation to hypoxia involves oxygen-sensing by a variety of specialized cells including carotid body (CB) type I cells, pulmonary neuroepithelial body cells (NEB), pulmonary artery myocytes and fetal adrenomedullary chromaffin cells. Twenty-four hours of hypoxia upregulated 388 and downregulated 363 genes in the mouse CB [36]. Among them, a group of ion channels demonstrated an oxygen-dependent regulation resulting in an increased expression of PMCP Kir6.1 and Kcnn4 and a downregulation of the modulatory subunit Kcnab1. Acute hypoxia induces membrane potential depolarization by closing a specific set of PMPC thus triggering cellular responses. Molecular strategies have recently allowed the identification of the PMPC subunits expressed in oxygen-sensing cells. Several voltage-gated PMPC subunits comprising six transmembrane segments and a single pore domain (Kv1.2, Kv1.5, Kv2.1, Kv3.1, Kv3.3, Kv4.2 and Kv9.3) were reversibly blocked by hypoxia when expressed in heterologous expression systems. Additionally, the background PMPC subunit TASK-1, which comprises four transmembrane segments and two pore domains, was also shown to be involved in oxygen-sensing in peripheral chemoreceptors [85]. The activity of three Shaker PMPC (Kv1.3, Kv1.4, and Kv1.5), one Shaw channel (Kv3.4), and one inward rectifier PMPC (IRK3) was drastically inhibited by photoactivation of rose bengal, a classical generator of ROS - butyl hydroperoxide, another generator of ROS, antagonized the fast inactivation processes of Kv1.4 and Kv3.4 but did not alter other channels. Thus, different types of PMPC are differently modified by ROS. Regulatory beta subunits may play an important role in the modulation of Kv channel subunits by ROS [29].

5. ROS, NADPH oxidase and oxygen-sensing

5.1. Basic considerations

Molecules that change their chemical properties in direct dependence on the surrounding PO2 may mediate the first step in oxygen-sensing. It was postulated that a non-phagocytic NADPH oxidase isoform within CB type I cells functions as an oxygen sensor to regulate ion channel conductivity. Various NADPH oxidase isoforms composed of the subunits p22phox, gp91phox, p47phox, p40phox, p67phox and Rac1, 2 have to be considered as potential oxygen-sensor candidates. The orthologues (NOX1–5) make use of the gp91phox component [22]. An evolutionary more distinct group has been named Duox. Furthermore isoforms of p47phox and p67phox termed NOXO1 and NOXOA1 have also been characterized [15]. It is believed that the NADPH oxidase converts PO2 into a redox signal by producing ROS which subsequently activate different cellular responses. NADPH oxidase function may however not be limited to the CB since NADPH isoforms are widely expressed throughout the body, e.g. NOX1 in pulmonary vascular smooth muscle cells [42,106] or the neutrophil NOX2 in endothelial cells [41] and NEB [35]. The existence of cytochrome b558 of the NADPH oxidase detected in the rat CB tissue by light microscopy [24] was later verified by immunohistochemistry in type I cells as well in tissue macrophages within the CB [30].

5.2. ROS-HIF

NADPH oxidase activity is exquisitely controlled by Rac proteins and growth factors suggesting fine-tuning of its oxygen-sensing function by integration into major signaling pathways. Thus, the GTPase Rac1 which modulates NADPH-oxidase mediated ROS production is involved in HIF-1α stabilization and plasminogen activator inhibitor-1 (PAI-1) induction. Overexpression of a constitutively active Rac1 (RacG12V) in HepG2 cells reduced nuclear HIF-1α as well as PAI-1 mRNA levels in normoxia and hypoxia concomitantly with an increase in ROS production. In contrast, expression of a dominant-negative Rac1 (RacT17N) elicited opposite effects [39]. Similarly, stimulation of human umbilical vein endothelial cells with prostacyclin stabilized HIF-1α specifically under prolonged hypoxia by attenuating NADPH oxidase derived ROS production due to suppressed Rac1 and p47phox expression [21]. In contrast, other groups could not confirm the inhibitory effect of NADPH oxidase signaling on HIF but instead reported that ROS production by renal NOX4 is essential for full HIF-2α expression and vascular endothelial growth factor (VEGF) or glucose transporter 1 (Glut-1) expression in renal tumor cells [72]. Furthermore the GTPase Rac1, which stimulates NADPH oxidase activity, was activated by hypoxia and a dominant-negative Rac1 repressed hypoxia-dependent HIF1-α accumulation [50]. Similarly hypoxia increased NOX1 mRNA and protein expression in A549 cells which was accompanied by enhanced ROS generation and activation of HIF-1-dependent target gene expression [42].

6. ROS-PMPC

Gp91phox knock out mice showed an impaired hypoxic ventilatory control in neonatal animals due to a decreased oxygen sensitivity of NEB PMPC conductivity [35,57] which provides evidence for an oxygen-sensor function of the NADPH oxidase in this oxygen-sensing cell type [86]. On the other hand gp91phox knock out mice did not reveal
an impairment in oxygen-sensing function of pulmonary vascular smooth muscle cells [6] or CB hypoxic drive [47,92]. However p47phox knock out mice demonstrated an enhanced CB hypoxic drive suggesting a distinct NOX isoform for the CB [93]. Under hypoxia, CB type I cells of p47phox knock out mice showed a significantly higher reduction of the potassium current and a significant higher intracellular calcium peak when compared with wild type animals. This response was not further enhanced by NADPH oxidase inhibition. External application of H$_2$O$_2$ reversed this hypoxia-induced response [48]. In HEK293 cells expressing NOX4 endogenously the TASK-1 activity was moderately inhibited by hypoxia, and this oxygen response was significantly augmented by NOX4. Moreover, the oxygen sensitivity of TASK-1 was abolished by NOX4 siRNA and NADPH oxidase inhibitors [64]. Studies on hypoxic pulmonary vasoconstriction (HPV) in p47phox knock out mice revealed that NADPH oxidase activity predominates in the acute phase [107]. Thus, various cell-specific NADPH oxidase isoforms may act as part of the oxygen-sensing system under hypoxia triggering the inhibition of PMPC to increase the calcium influx into oxygen-sensing cells [102].

7. ROS, mitochondria and oxygen-sensing

7.1. Basic considerations

The mitochondrial ETC has been implicated in oxygen-sensing function. As such, cytochrome c oxidase (cytochrome aa$_3$, complex IV) is considered to be the CB oxygen sensor [75,110]. Succinate dehydrogenase D (SDHD) of mitochondrial complex II was proposed as an oxygen sensor for the CB [8] and pulmonary vasculature [82]. However, SDHD$$^-^-^-$$ mice did not show an impairment of the oxygen-sensing property of CB type I cells questioning the role of SDHD as an oxygen sensor [87]. An oxygen-sensor role of mitochondrial complex IV was strongly suggested for the sustained phase of HPV [107]. Metabolic responses of mammalian cells toward declining oxygen concentrations are generally thought to occur when oxygen limits mitochondrial ATP production at a $P_{O2}$ below 1 mm Hg [7]. Consequently, the mitochondrial chain sensor would only operate at a low $P_{O2}$ within a small sensing range, a suboptimal prerequisite for an oxygen sensor. However, various affinity modulators have been proposed that might convert the mitochondrial ETC from high into intermediate $P_{O2}$ affinity systems to allow sensing over wider tissue $P_{O2}$ changes. The CB complex IV may have an intermediate $P_{O2}$ affinity due to a mutated, low midpoint potential cytochrome $a$ (cytochrome $a592$) lowering the high $P_{O2}$ affinity of cytochrome $c$ oxidase to about 30 mm Hg [97]. Similarly, ROS and NO have been demonstrated to apparently lower ETC affinity. At $P_{O2}$ levels markedly above those limiting to mitochondria several mammalian cell types already displayed reduced rates of oxygen consumption [77]. Oxygen consumption was 31% greater at high as compared to low $P_{O2}$, concomitantly with elevated mitochondrial ROS production, partly due to energy wastage through mitochondrial proton leakage and elevated intracellular calcium turnover. Mitochondria derived H$_2$O$_2$ was implicated in this response because catalase prevented the increase in oxygen consumption whereas H$_2$O$_2$ generated by exogenous glucose oxidase enhanced oxygen consumption [77]. Thus, ambient oxygen concentrations would be translated into a mitochondria derived ROS signal regulating mitochondrial oxygen consumption and respiratory electron flux at $P_{O2}$ levels markedly above 1 mm Hg. By reducing mitochondrial oxygen consumption in response to hypoxia the declining ROS levels would act as a key modulator of mitochondrial function helping to flatten the decline in oxygen availability and delay the induction of hypoxic cell responses. Similarly, inhibition of mitochondrial respiration by NO via complex IV under low oxygen tension resulted in reduced mitochondrial oxygen consumption. This obviously leads to a redistribution and increased availability of intracellular O$_2$ reactivating prolyl hydroxylation of HIF-$$\alpha$$ subunits [46]. The sensitivity of 2-oxoglutarate and succinate dehydrogenase to ROS-mediated inactivation and the reversible nature of inactivation is another example for the potential role of ROS in the modulation of mitochondrial ETC $P_{O2}$ affinity [79]. Recently, it was reported that NO increases mitochondrial ROS formation [18,37] by modifying the redox state of complex IV [83] linking the impact of NO and ROS on mitochondrial oxygen-sensing. However, other groups excluded a redox modulation by NO [46].

Mitochondria produce ROS under physiological conditions with complex I releasing superoxide on the matrix side of the inner membrane and complex III on the cytoplasmic side. However, only 0.15% of the electrons flowing through ETC give rise to mitochondrial ROS formation which is more than an order of magnitude lower than commonly cited values of 1–2% [96]. Several reports document that the decreasing mitochondrial membrane potential as it occurs under hypoxia [28] is associated with a reduction in mitochondrial ROS formation [18,56,63] whereas other groups demonstrate enhanced mitochondrial ROS production with decreasing O$_2$ levels [17,43,71]. Complex III was suggested to act as the major source of ROS production by transferring an electron via the ubiquinol cycle to molecular O$_2$ yielding ROS.

7.2. ROS-HIF

It was reported that a functional mitochondrial complex III as well as an increase in mitochondrial ROS formation upon hypoxia was required for the hypoxic stabilization of HIF-1$$\alpha$$ and HIF-2$$\alpha$$. This finding was based on the fact that HIF-1$$\alpha$$ was increased by impairing mitochondrial
electron flow by chemical ETC inhibitors, use of mitochondria deficient ρ0 cells or interference with electron transfer to complex IV using cytochrome c deficient ES cells or siRNA mediated knock-down of Rieske iron–sulfur protein [17,43,71]. In contrast, other groups demonstrated that a functional mitochondrial respiratory chain may not be necessary for HIF-1α activation [27,95,99]. Intracellular hypoxia detection by pimonidazole staining and HIF activity were substantial in 0.1% O2 irrespective of ETC inhibition. In contrast, at 3% O2 pimonidazole staining and HIF-1α expression were detectable but strongly reduced after ETC inhibition in conventional cell cultures. These results indicate that intracellular oxygen levels were elevated following the decrease in mitochondrial oxygen consumption suggesting that the increased availability of oxygen for PHD-function was responsible for the degradation of HIF-1α [46]. The impact of PO2 gradients in cell culture dishes was confirmed by a previous study demonstrating that when cells were subjected to decreasing oxygen tensions in the absence or presence of KCN the increases in HIF-1α–DNA-binding activity and HIF-1α levels was almost identical at low oxygen levels. Using a tonometer to optimize oxygen supply in suspension cultures of Hela cells, the oxygen gradient formation of conventional adherent cell culture was minimized in these experiments [55]. The same effect was achieved by culturing cells on gas permeable membranes [27]. Elevations in the PO2 gradient might also explain why inhibitors and uncoupling of the mitochondrial ETC significantly abrogated hypoxia-induced HIF-1α expression in isolated rat CB type I cells [9]. Treatment with succinate, a substrate for complex II, abolished the HIF-1α destabilizing effect of complex I inhibition by rotenone during hypoxia probably by increasing oxygen consumption and steeping PO2 gradients. Inhibition of pyruvate dehydrogenase (PDH) by HIF induced upregulation of pyruvate dehydrogenase kinase 1 (PDK1) seems to result in a decreased mitochondrial ROS production under hypoxia due to decreased oxygen consumption [60,84].

8. ROS-PMPC

Background PMPC leading to membrane depolarization and voltage-gated calcium entry of rat CB type I cells were reduced by ETC inhibitors as well as uncouplers and abolished oxygen sensitivity of these channels [112]. This might hint to the unique properties of the CB ETC with a low PO2 affinity complex IV [97] controlling intracellular calcium levels by regulating ETC membrane potential [28]. External application of H2O2 was found to inhibit the time-dependent fast inactivation process of potassium currents of KShIIIC and KShIIID voltage-gated PMPC, which is similar to the O2-sensitive PMPC present in CB type I cells. The effects of H2O2 were specific and reversible [100]. These results were supported by the excitatory effect of iron chelators on CB nervous activity indicating that a Fenton reaction controlling PMPC open probability is involved [91]. Rat pulmonary smooth muscles exhibited a closing of Kv channels upon decreased mitochondrial ROS formation and an increase in intracellular calcium with elevated mitochondrial ROS levels. Bovine pulmonary smooth muscle cells showed a decrease in cGMP induced by a decrease in NOX-derived ROS. A decreased cytosolic NADPH activity lowered intracellular calcium in bovine coronary smooth muscle cells. An increased mitochondrial ROS formation opened PMPCs in renal arterial smooth muscle cells [111]. In case of a hypoxia induced fall in cellular ATP a concomitant rise in the AMP/ATP ratio would activate an AMP-activated protein kinase with subsequent calcium mobilisation from the ER in pulmonary arterial myocytes or transmembrane calcium influx into CB type I cells [33].

9. ROS, PHDs and oxygen-sensing

9.1. Basic considerations

Interaction of pVHL with HIF-α requires an O2- and iron-dependent hydroxylation of specific prolyl-residues (Pro 402, Pro 564 for human HIF-1α) within the HIF-1α ODD (oxygen-dependent degradation domain) by PHDs [16,32]. So far, four orthologues of PHDs have been described (PHD1–3 and PH4), although PH4 has formally not been demonstrated to hydroxylate HIF-α [80]. PHDs are considered as oxygen sensors with a prominent sensitivity to graded levels of oxygen in vitro, mirroring the progressive increase in HIF-α protein abundance [32]. In line with this observation, PHDs have a strikingly low O2 affinity with a Km of 178 mm Hg which is above the concentration of dissolved O2 in the air [52]. How accurately these in vitro measurements obtained with a short HIF-α peptide reflect the in vivo situation remains to be determined. Consequently, PHDs would operate in the tissue under non-equilibrium conditions for HIF-α turnover far below their Km. However, given a regular Michaelis-Menten kinetic this would allow the enzymes to operate in a highly sensitive manner, in which already small changes in oxygen concentration result in significant changes of enzymatic activity and, thus HIF-α turnover.

A second oxygen dependent switch involves hydroxylation of an asparagine residue within the C-TAD of HIF-1α subunits by FIH-1 [62]. Asparagine hydroxylation apparently interferes with recruitment of the coactivator p300 resulting in reduced transcriptional activity. FIH was shown to have a Km for O2 of around 64 mm Hg, suggesting that this enzyme also acts as a oxygen sensor at least under conditions found in normoxic tissues in vivo [65]. Both PHDs and FIH belong to a superfamily of 2-oxoglutarate dependent hydroxylases which employ non-heme ferrous
iron in the catalytic moiety [49]. One oxygen atom of dioxygen is incorporated in the prolyl or asparagyl residue, respectively, and the other into 2-oxoglutarate yielding succinate and CO₂. Thus, the hydroxylation reaction is inherently dependent on ambient oxygen pressure, providing a molecular basis for the oxygen-sensing function of these enzymes.

PHD2 and PHD3 expression is upregulated by hypoxia though the degree of induction apparently varies between cell type and P\textsubscript{O₂} analyzed [13,25,32,74]. PHD3 is coordinately expressed with known HIF target genes, while induction of PHD2 by hypoxia has HIF-1-independent and -dependent components [5]. Short-term hypoxia resulted in induction of PHD2 independent of HIF-1, while PHD2 accumulation by prolonged hypoxia was HIF-1α dependent. siRNA mediated knock-down studies suggest that PHD2 is the rate-limiting enzyme controlling the steady-state levels of HIF-1α in normoxia at least under cell culture conditions [13]. Interestingly, a role for PHD1 in controlling HIF-1α levels under long-term maintenance of hypoxia (5–6 days) was proposed suggesting further non-redundant functions of each PHD orthologue in different physiological or pathophysiological settings. This notion was further supported by experiments documenting distinct functions of the PHD orthologues on different HIF isoforms with PHD2 predominantly regulating HIF-1α and PHD3 HIF-2α protein levels depending on the relative expression levels of each PHD orthologue [4].

OS-9 [10] and Siah2 [78] are additional proteins controlling HIF or PHD1/PHD3 half-life, respectively. Furthermore, in addition to regulating HIF stability, HIF prolyl-hydroxylases can modulate HIF function through the recruitment of tumor suppressor protein inhibitor of growth family member 4 (ING4), a likely component of a chromatin-remodeling complex [81]. Finally a delicately balanced ratio between HIF-α and PHDs controlling each other in an auto-regulatory loop has a great impact on the final outcome of hypoxia induced gene upregulation [5].

9.2. ROS-PHD function

Different and opposing effects on PHD function and, consequently, HIF stabilization have been reported. A recent study suggests that the effect of ROS on PHD function may be concentration dependent although the underlying mechanism was not elucidated [18]. High ROS concentrations blocked PHD activity and subsequently stabilized HIF expression, at least under normoxic conditions. This is in line with previous studies showing that ROS increase in response to various hormones, cytokines and or growth factors and induce HIF stabilization [45,90] possibly via enhanced NADPH-oxidase formation [40]. Recent studies suggest that ROS may influence co-factor concentrations needed for PHD function. Apart from PO₂, PHD activity was regulated by the amount of ferrous iron recovered by antioxidants such as vitamin C [61]. In fact, the accumulation of H₂O₂ following JunD deficiency promoted oxidation of iron, thus depleting PHD of an essential cofactor [38]. In addition, ROS have been shown to interfere with mitochondrial function at the level of 2-oxoglutarate dehydrogenase and succinate dehydrogenase, which may influence 2-oxoglutarate and succinate levels [79]. Interestingly, both succinate and fumarate, sequential metabolites of the mitochondrial Krebs cycle, have been shown to increase HIF-1α protein levels by PHD inhibition providing an additional mechanism on how ROS may block PHD activity [54,94]. In contrast, low-level ROS formation was shown to increase PHD activity under normoxia and hypoxia [18] in line with a previous report demonstrating that the localized ER-based OH⁻ formation mediated by the Fenton reaction affects HIF-1α degradation. Inhibition of the Fenton reaction by the OH⁻ scavenger DHR attenuated HIF-prolyl-hydroxylase activity and interaction with von-Hippel-Lindau protein, leading to enhanced HIF-1α levels [66]. Up to now, the mechanism on how ROS induce PHD activation has not been elucidated.

In consequence, HIF and PHD are regulated in an iron- and redox-sensitive fashion. It is interesting to note that other redox regulatory systems have been shown to impact on HIF-α activity by regulating HIF-α stabilization and transactivation function. The implication of iron in oxygen-sensing by 2-oxoglutarate dependent hydroxylases and the involvement of iron through the Fenton reaction [88] generating ROS makes the interaction between oxygen and iron redox state physiologically highly important for oxygen-sensing.

10. ROS, heme oxygenase-2 and oxygen-sensing

10.1. Basic considerations

In the presence of oxygen and NADPH, HO-2, constitutively expressed in tissue, is responsible for the oxidation of cellular heme and generates CO, iron and biliverdin [69]. HO-2 was localized to glomus cells in the cat and rat CB as shown by immunocytochemistry. Physiological studies show that zinc protoporphyrin IX, a potent heme oxygenase inhibitor, markedly increases CB sensory activity, while copper protoporphyrin IX, which does not inhibit the enzyme, was inactive. Exogenous CO reversed the stimulatory effects of zinc protoporphyrin IX [89].

10.2. ROS-PMPC

The large conductance, voltage- and calcium-dependent potassium oxygen-sensing channel BK\textsubscript{Ca} identified in CB type 1 cells seems to be closely linked to HO-2 which controls the channel conductance by a PO₂ dependent CO production [109]. HO-2 might not only decrease CO production under hypoxia but also lead to a diminished iron generation impairing the Fenton reaction with subse-
sequent inhibition of PMPC. This suggestion is substantiated by the inhibitory effects of iron chelators on PMPC open probability with subsequent excitatory effect on CB nervous activity indicating that a Fenton reaction is involved in CB oxygen-sensing [91].

10.3. Synopsis

How can one reconcile the different candidates and concepts of oxygen-sensing? We propose that the oxygen-sensor candidates NADPH oxidase, mitochondrial ETC, PHD/FIH and HO-2 with their different \( \mathrm{O}_2 \) affinities cooperate to match the heterogeneous tissue \( \mathrm{PO}_2 \) distribution. Three putative scenarios are outlined in a hypothetical scheme (see Fig. 1) on how ROS, whether decreasing \([18,19,39,66,105]\) or increasing \([20,42,43,48,50,71]\) upon hypoxia, might interfere with the oxygen-sensing pathway to regulate HIF stabilization and transcriptional activity as well as PMPC conductivity.

10.3.1. ROS formation under hypoxia is decreased

Due to the low \( \mathrm{PO}_2 \) affinity of PHD and FIH a declining \( \mathrm{O}_2 \) concentration instantly and progressively impairs enzymatic hydroxylation of HIF-\( \alpha \) subunits resulting in enhanced HIF-\( \alpha \)-protein stabilization and transactivation. At the center of this scenario the NAD(P)H oxidase as the major ROS generator converts the \( \mathrm{PO}_2 \) into a redox signal. Thus, when \( \mathrm{O}_2 \) levels approach 20 mm Hg, the Km of NAD(P)H-oxidase, ROS formation by this oxygen sensor is drastically reduced. Via a yet unidentified mechanism the concomitant fall in \( \mathrm{PO}_2 \) and ROS leads to the inactivation of PHDs \([18,66]\) and/or to recruitment of \( \mathrm{p300} \) to the C-terminal transactivation domain of HIF with subsequently enhanced gene expression. The participation of a Fenton reaction, which is probably fueled with iron by HO-1, was enhanced gene expression. The participation of a Fenton reaction, which is probably fueled with iron by HO-1, was discussed [66]. The activation of this second oxygen-sensor system may help to explain the exponential induction of HIF by oxygen-sensing activity [98].

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Additionally, mitochondria may interface by shaping ROS production and \( \mathrm{O}_2 \) gradients. In case of a high \( \mathrm{PO}_2 \) affinity mitochondrial oxygen-sensor system, mitochondrial ETC oxygen consumption is unchanged down to very severe hypoxia with unchanged membrane potential as well as ROS production. However, the mitochondrial \( \mathrm{PO}_2 \) affinity is likely to be affected by ligands like NO or ROS [18,46,77]. In case of an intermediate \( \mathrm{PO}_2 \) affinity mitochondrial oxygen-sensor [97] mitochondrial ETC oxygen consumption, ROS production and membrane potential gradually decrease [77], impacting on intracellular calcium levels regulating neurotransmitter release, PMPC activity and HIF stabilization [12,67,113].

Two mechanisms may partially counteract adaptive responses towards the decreased tissue \( \mathrm{PO}_2 \). First, the decreased PHD activity at lower \( \mathrm{PO}_2 \) levels may to some extent be compensated for by the increased availability of ferrous iron due to the more reduced state of the cell. At least in cancer cells the amount of ferrous iron was limiting for PHD activity [61]. Second, a decrease in oxygen consumption mediated by reduced mitochondrial ROS production [77] or PDH activity [60,84] may flatten the decline in \( \mathrm{PO}_2 \) levels and increase intracellular oxygen availability for prolyl hydroxylation of HIF-\( \alpha \) subunits [27,46].

10.3.2. ROS formation under hypoxia is increased

Enhanced electron flow at complex III of mitochondrial ETC [17,43,71] and NADPH oxidase [42] have been identified as sources for an augmented ROS formation under hypoxia aiding HIF stabilization. Though the underlying mechanisms how ROS may impact on the HIF pathway under hypoxia have not yet been elucidated, recent findings suggest that ROS may impair PHD activity by promoting the ferric iron state [38], change 2-oxoglutarate/succinate levels by interfering with mitochondrial function [79] or directly control the phosphorylation state of HIF by activation kinases and/or inactivation of phosphatases [44,58]. In addition, the change in energy metabolism may augment HIF activation. ROS and hypoxia reduce mitochondrial ETC activity followed by enhanced glycolysis. The glucose metabolites pyruvate and oxaloacetate specifically reduced HIF-1\( \alpha \) destabilization by binding to the 2-oxoglutarate site of PHDs [68]. Subsequent lactate production and intracellular acidification elicits a transient and reversible loss of pVHL function through nuclear sequestration promoting HIF-1\( \alpha \) stabilization even under normoxia [73]. PMPC will react according to their molecular structure specifically to the increase in ROS.

10.3.3. ROS formation under normoxia is increased

Extracellular signals like hormones, cytokines and/or growth factors increase NAD(P)H oxidase-mediated ROS formation [40] followed by HIF stabilization [45,90]. A further complicating forward loop is induced by the ROS mediated activation of p38MAPK and PI3 kinase/protein kinase B [26] and phospholipase A\( \_2 \) [23] followed by an upregulation of the NADPH oxidase subunit p22phox and subsequent ROS formation. Similarly as depicted in scenario 2, ROS may interfere with PHD activity by alteration of co-factor concentrations inducing iron oxidation as evidenced for JunD [38] and changing 2-oxoglutarate/succinate/pyruvate/oxalacetate levels. Though this way of HIF activation may not directly be part of an oxygen signaling response, the integration of oxygen-sensing mechanisms into major metabolic signaling pathways may help to set and tailor the various adaptive and dynamic responses of different cell types and differentiation stages according to tissue oxygen availability.
Impaired tissue oxygenation is implicated with the pathophysiology of diseases such as myocardial infarction, stroke and cancer, the leading causes of death in Western societies. Thus, deeper understanding of how cell sense and react to changes in oxygen tension may give clues on how to open new therapeutic avenues. In a number of studies the decisive role of the hypoxia-inducible transcription factors HIF-1α and HIF-2α in defining the tumor phenotype by eliciting pro-tumorigenic mechanisms has been defined. However, a recent study [3] indicates that HIF may also have a tumor-suppressive role suggesting a dual function of HIF in tumor biology [2]. Thus, though decreasing tumor vascularization, HIF inhibition using a dominant-negative HIF transgene in gliomas or HIF-2α deficiency in teratomas accelerated tumor growth partly due to a decrease in hypoxia induced tumor apoptosis. Moreover, HIF-1α crucially determines tumor responsiveness to radiotherapy [76]. By promoting ATP metabolism, proliferation, and p53 activation, HIF-1α has a radio-sensitizing effect on tumors. In contrast, through stimulating endothelial cell survival, HIF-1α has a radio-sensitizing effect on tumors. However, a recent study [3] indicates that HIF may also have a tumor-suppressive role suggesting a dual function of HIF in tumor biology [2]. Thus, though decreasing tumor vascularization, HIF inhibition using a dominant-negative HIF transgene in gliomas or HIF-2α deficiency in teratomas accelerated tumor growth partly due to a decrease in hypoxia induced tumor apoptosis. Moreover, HIF-1α crucially determines tumor responsiveness to radiotherapy [76]. By promoting ATP metabolism, proliferation, and p53 activation, HIF-1α has a radio-sensitizing effect on tumors. In contrast, through stimulating endothelial cell survival, HIF-1α promotes tumor radio-resistance. Thus, employing HIF inhibitors as cancer therapeutic strategies requires careful consideration and characterization of pro- and antitumorigenic functions of the oxygen-sensing pathway.

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